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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	
Satoshi SAITO et al.	Group Art Unit: 1633
Application No.: 10/507,129	Examiner: Scott Long
Filing Date: April 11, 2005	Confirmation No.: 8898
For: METHOD OF CONTROLLING ETHANOL PRODUCTION AND MASS PRODUCTION OF LACTIC ACID AND TRANSFORMANT THEREFOR	

Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

DECLARATION UNDER 37 C.F.R. § 1.132

I, Toru ONISHI, do hereby make the following declaration:

1. My name is Toru ONISHI, and I received a Master's degree in Molecular Biology from Nara Institute of Science and Technology in Nara, Japan in 1996. I have been engaged in research and development in the fields of molecular biology, microbiology, and biotechnology for thirteen (13) years. I am currently employed as an Assistant Manager in Toyota Jidosha Kabushiki Kaisha, in Aichi, Japan. I am very familiar with methods of controlling ethanol production and mass production of lactic acid, and transformant therefor.

2. I have read and understood the above-identified U.S. Patent Application No. 10/507,129, filed April 11, 2005 ("the '129 application"), including the specification, drawings, and claims.

3. I have read and understood the final Office Action mailed in the '129 application on November 23, 2009 from the United States Patent and Trademark Office.

4. I have read and understood the amendments to the claims presented in the Reply to Office Action under 37 C.F.R. § 1.114. I understand that these amendments to the claims are being filed with this Declaration under 37 C.F.R. §1.132.

5. I have read and understood the following references cited in the present application at paragraph [0003] and the final Office Action:

- Porro et al., *Development of metabolically engineered* Saccharomyces cerevisiae *cells for the production of lactic acid,* Biotechnol. Prog. Vol. 11, No. 3, pp. 294-298 (1995). ("Ref. 1"); and
- Adachi et al., *Modification of metabolic pathways of* Saccharomyces cerevisiae *by the expression of lactate dehydrogenase and deletion of pyruvate decarboxylase genes for the lactic acid fermentation at low pH value*, J. Ferment. Bioeng. Vol. 86, No. 3, pp. 284-289 (1998). ("Ref. 2"); and
- Porro et al., PCT International Application Publication No. WO 99/14335 (1999) ("Porro").

6. I have read and understood the following references accompanying my testimony below (copies attached):

- Ishida, N. et al., *Efficient production of L-Lactic acid by metabolically engineered* Saccharomyces cerevisiae *with a genome-integrated L-lactate dehydrogenase gene*. Appl. Environ. Microbiol. Vol. 71, No. 4, pp. 1964-1970 (2005). ("Ref. 3"); and
- Current Protocols in Molecular Biology, Chapter 13, Unit 13.4 (John Wiley & Sons, January, 1993) [Online] http://www.currentprotocols.com/protocol/mb1304 (Online Posting Date: May, 2001). ("Ref. 4").

7. Given my education and experience, particularly in the area of methods of controlling ethanol production and mass production of lactic acid and transformant therefor, I consider myself able to provide the following testimony.

8. Each of the above-mentioned Refs. 1-3, Porro, and the '129 application discloses comparative tests for production of lactic acid using bacterial or yeast transformants carrying a lactate dehydrogenase gene (LDH), as summarized in Table 1 below.

9. In each test, production of lactic acid was measured under substantially similar experimental conditions, e.g., types of fermentation medium and fermentation temperature range.

Table 1

	Ref. 1	Ref. 2	Ref. 3		present application	WO 99/14335
Figure or table	Fig. 4	Fig. 5; Table 1	Table 3; Figs. 3 and 4		Table 2	Tables A and 3A
LDH gene type	Bovine	Bovine	Bovine	Bovine	Bovine	L. casei
Methods of expression for LDH	2μm plasmid; GAL/CYC promoter	2μm plasmid; ADH1 promoter; PDC1,5,6 genes destroyed	2μm plasmid, PDC1 promoter; PDC1 gene destroyed	Introducing LDH on chromoso me; PDC1 gene destroyed	Introducing LDH on the chromoso me; PDC1 gene destroyed	2μm plasmid; TPI promoter
Initial conc. of glucose	62 g/L	= 48 g/L (Fig. 5)	100 g/L	100 g/L	150 g/L	88.4 g/L
lactic acid production	≈12.0 g/L (Fig. 4)	5.63 g/L	16.3 g/L	55.6 g/L	49.2 g/L	29.5 g/L
lactic acid yield *	≈ 22% (Fig. 4)	12.3%	19.2%	62.2%	32.8%	33.8%
Fermentation medium	YNB-GLU based medium	minimal medium	YPD10 medium	YPD10 medium	YPD medium	rich medium
Fermentation temperature	30 °C	30 °C	30 °C	30 °C	30 °C	30 °C

* Yields are expressed as grams of lactate produced per liter divided by grams of glucose consumed per liter.

10. In Ref. 1, 2 μm plasmid carrying a bovine LDH gene under control of GAL/CYC promoter was incorporated into *S. cerevisiae*. Similarly, in Ref. 2, 2 μm plasmid carrying a bovine LDH gene under control of ADH1 promoter was incorporated into *S. cerevisiae*. The yields of lactic acid production were approximately 22% in Ref. 1 (see Fig. 4) and 12.3% in Ref. 2 (see Fig. 5).

11. In Porro, 2 μm plasmid carrying an *L. casei* LDH gene under control of TPI promoter was incorporated into *S. cerevisiae*. The yield of lactic acid production was 33.8% (see Table 3A).

12. In the '129 application, a single copy of an LDH gene was inserted into each haploid chromosome in *S. cerevisiae* and expressed under control of PDC1 promoter The yield of lactic acid production was 32.8% (see Table 2, strain KCB-27).

13. Ref. 4 at 4th paragraph discloses that a plasmid carrying 2μm sequence ("2μm plasmid") maintains high copy number (20 to 50 copies). In addition, in Ref. 1 and 2, an appropriate medium (YNB-GLU based medium in Ref. 1 and minimal medium in Ref. 2) was chosen to prevent the 2μm plasmid from being lost during culture (see Materials and Methods section and Fig. 2 of Ref. 1; Materials and Methods section of Ref. 2). Porro at pages 30 and 31 also discloses that the combination of the yeast strain and plasmid transfection backbone was known to produce a stable transfection, preventing loss of plasmids into the cell culture medium.

14. In view of the above teachings, in Refs. 1, 2, and Porro, each transformed yeast cell maintains multiple copies of an LDH gene because each transformant would contain multiple copies of the 2μm plasmid. In contrast, the transformant according to the claims of the '129 application maintains a single copy of an LDH gene incorporated into the chromosome.

15. Therefore, the claimed transformants according to claims 1 and 16, and their dependent claims, containing a single copy of an LDH gene incorporated into the chromosome, produced lactic acid in the amount larger than, or at least similar to, the amount of lactic acid produced from the yeast transformants carrying multiple copies of an LDH gene, as disclosed in Refs. 1 and 2, and Porro. In summary, the claimed transformants showed an increased efficiency of lactic acid production from introducing

only a single copy of an LDH gene compared to the yeast cells introducing multiple copies of an LDH gene.

16. Ref. 3 also corroborate the above-discussed beneficial results. In Ref. 3, the differences in lactic acid production were compared between a transgenic strain with 2um plasmid (YEBO-1B) and a genome-integrated strain with integration-type vector (YIBO-1A) (Table 3 and 4 of Ref. 3). The results indicate that yield of L-lactic acid was higher in yeast cells introducing an LDH gene under the control of the PDC1 promoter on the chromosome than the yeast cells introducing multiple LDH gene copies with the 2μm plasmid.

17. The above-mentioned beneficial results in the '129 application was unexpected in the cited prior art. For example, Porro discloses that an increased LDH activity of the transformed yeast cell as a consequence of an increase in the copy number of LDH genes per cell using high copy number plasmids such as 2um plasmid (see pages 5, 6, 12 and 54 in the specification). In view of the prior art teaching, one of ordinary skill in the art, at the time of invention, would recognize an increased efficiency of lactic acid production due to introducing multiple copies of an LDH genes to the cells. Refs. 1 and 2, and Porro, provide no reason to expect an increased efficiency of lactic acid production from the chromosomally-integrated LDH gene. One of ordinary skill in the art at the time of invention, therefore, would not have had expected successful results from introducing a single copy of an LDH gene to a host cell.

18. I declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: 17/03/2010 By: Toru Onishi
Toru ONISHI